Clostridium cellulovorans metabolism of cellulose as studied by comparative proteomic approach

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Corresponding Author: Dr. Roberto Mazzoli, Ph.D.

Corresponding Author's Institution: University of Torino

First Author: Giulia Usai

Order of Authors: Giulia Usai; Simona Cirrincione; Angela Re; Marcello Manfredi; Andrea Pagnani; Enrica Pessione; Roberto Mazzoli, Ph.D.

Abstract: Clostridium cellulovorans is among the most promising candidates for consolidated bioprocessing (CBP) of cellulosic biomass to liquid biofuels (ethanol, butanol). C. cellulovorans can metabolize all the main plant polysaccharides and its main catabolite is butyrate. This makes this strain a potential butanol producer since most reactions of butyrate and butanol biosynthesis from acetyl-CoA are common. Recent studies demonstrated that introduction of a single heterologous alcohol/aldehyde dehydrogenase diverts the branching-point intermediate, i.e. butyryl-CoA, towards butanol production in this strain. Despite C. cellulovorans potential for CBP of plant biomass, engineering its metabolic pathways towards industrial utilization requires better understanding of its metabolism. The present study aimed at improving comprehension of cellulose metabolism in C. cellulovorans by comparing growth kinetics, substrate consumption/product accumulation and whole-cell soluble proteome (data available via ProteomeXchange, identifier PXD015487) with those of the same strain grown on a soluble carbohydrate, glucose, as the main carbon source. Modulations of the central carbon metabolism in response to different growth substrate were detected, including regulation of glycolytic enzymes, fermentation pathways and nitrogen assimilation possibly affecting the redox balance. Higher energy expenditure seems to occur in cellulose-grown C. cellulovorans, which induces up-regulation of ATP synthetic pathways, e.g. acetate production and ATP synthase.

Suggested Reviewers: Daniel G. Olson PhD
Research assistant professor, Thayer School of Engineering, Dartmouth college, Hanover, NH 03755, USA
Daniel.G.Olson@dartmouth.edu
Expertise in the metabolism and metabolic engineering of anaerobic cellulolytic bacteria

Adam M. Guss Ph.D.

Assistant Professor, Biosciences Division, Oak Ridge National Laboratory, One Bethel Valley Road, Oak Ridge, TN 37831, USA
gussam@ornl.gov
Expertise in the metabolism and metabolic engineering of anaerobic cellulolytic bacteria

David B. Levin
Professor, Department of Biosystems Engineering, University of Manitoba, Winnipeg R3T 2N2, MB, Canada.
david.levin@umanitoba.ca
Expertise in proteomics and metabolism of anaerobic cellulolytic bacteria

Shang-Tian Yang
Professor, Department of Chemical and Biomolecular Engineering, The Ohio State University, 151 West Woodruff Avenue, Columbus, OH, 43210, USA
yang.15@osu.edu
Expertise in metabolic engineering of Clostridium cellulovorans

Vincent J.J. Martin
Professor, Centre for Structural and Functional Genomics, Concordia University, 7141 Sherbrooke Street West, Montréal, QC H4B 1R6, Canada
vincent.martin@concordia.ca
Expertise in proteomics and metabolic engineering aimed at biorefinery of cellulosic biomass

Ariane Bize
Research director, UR HBAN, Irstea, Antony, France
ariane.bize@irstea.fr
Expertise in proteomics of anaerobic cellulolytic bacteria

Opposed Reviewers:

Cover letter

Dear Editor,

please find attached the manuscript entitled "*Clostridium cellulovorans* metabolism of cellulose as studied by comparative proteomic approach". *C. cellulovorans* is among the most attractive candidates for direct fermentation of lignocellulosic biomass to industrially relevant chemicals, particularly, biofuels. Recently, metabolic engineering has enabled butanol production in this strain, which is a milestone in one-step production of this biofuel from plant biomass. However, currently scarce information on the central carbon metabolism of *C. cellulovorans* hampers further implementation of metabolic engineering strategies towards application of this strain in industrial processes. The present study aimed at improving understanding of cellulose metabolism in *C. cellulovorans*. Growth kinetics, substrate consumption, catabolite accumulation and whole-cell soluble proteome of cellulose-grown cells were compared with those of glucose-grown cells. This analysis showed specific modulations of the central carbon metabolism in response to changes in the growth substrate, including modifications in the redox and energy balance. We think that the results of the present study will help better understanding of *C. cellulovorans* physiology. Furthermore, these data could be useful to determine key genes and possible metabolic bottlenecks to be addressed towards improved metabolic engineering of *C. cellulovorans*.

We hope that the subject and of the present manuscript will be of interest for publication in Journal of Proteomics. We look forward to receive your answer and we send you our best regards.

Sincerely yours,

Roberto Mazzoli

Significance

C. cellulovorans can metabolize all the main plant polysaccharides (cellulose, hemicelluloses and pectins) and, unlike other well established cellulolytic microorganisms, can produce butyrate. *C. cellulovorans* is therefore among the most attractive candidates for direct fermentation of lignocellulose to high-value chemicals and, especially, n-butanol, i.e. one of the most promising liquid biofuels for the future. Recent studies aimed at engineering n-butanol production in *C. cellulovorans* represent milestones towards production of biofuels through one-step fermentation of lignocellulose but also indicated that more detailed understanding of the *C. cellulovorans* central carbon metabolism is essential to refine metabolic engineering strategies towards improved n-butanol production in this strain. The present study helped identifying key genes associated with specific catabolic reactions and indicated modulations of central carbon metabolism (including redox and energy balance) associated with cellulose consumption. This information will be useful to determine key enzymes and possible metabolic bottlenecks to be addressed towards improved metabolic engineering of this strain.

*Graphical Abstract

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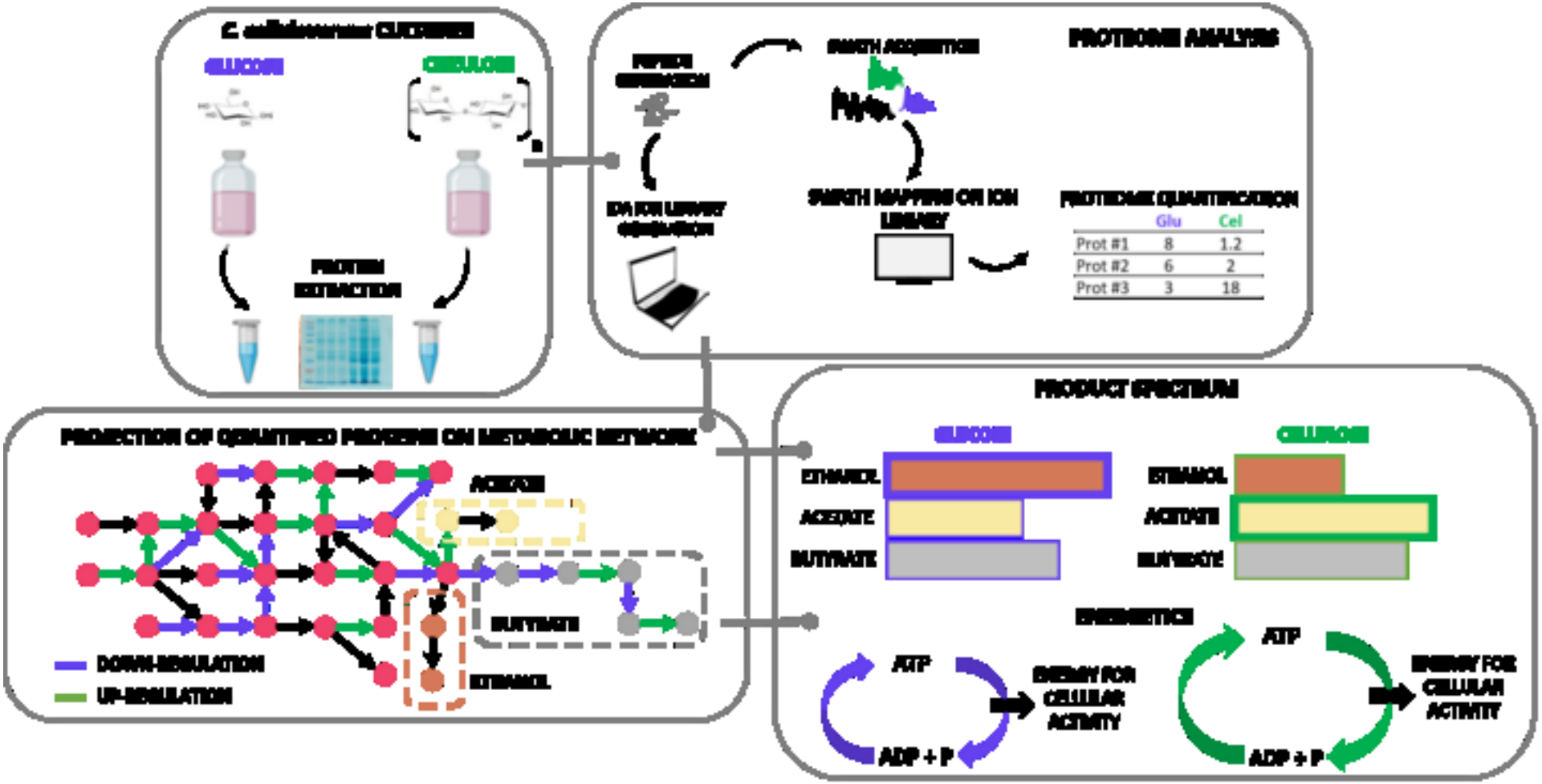

Highlights

- Whole-cell soluble proteome of cellulose- and glucose-grown *C. cellulovorans*
- Cellulose-grown cells produce higher amount of acetate and lower amount of ethanol
- Modulation of glycolysis, fermentative pathways, nitrogen assimilation was detected
- Cellulose induces up-regulation of ATP biosynthetic pathways
- Cellulose-grown cells show lower intracellular ATP content

Clostridium cellulovorans metabolism of cellulose as studied by comparative proteomic approach

Giulia Usai[1], Simona Cirrincione[1], Angela Re[2], Marcello Manfredi[3], Andrea Pagnani[4], Enrica Pessione[1], Roberto Mazzoli[1,#]

[1]Structural and Functional Biochemistry. Laboratory of Proteomics and Metabolic Engineering of Prokaryotes. Department of Life Sciences and Systems Biology, University of Turin. Via Accademia Albertina 13, 10123 Torino, Italy.

[2]Centre for Sustainable Future Technologies, Fondazione Istituto Italiano di Tecnologia, Environment Park - Parco Scientifico Tecnologico per l'Ambiente Via Livorno 60, 10144, Torino, Italy

[3]Center for Translational Research on Autoimmune and Allergic Diseases, Università del Piemonte Orientale, Novara, Italy; Department of Translational Medicine, Università del Piemonte Orientale, Novara, Italy.

[4]DISAT, Politecnico di Torino, Corso Duca degli Abruzzi 24, 10129 Torino, Italy. Italian Institute for Genomic Medicine (IIGM), Via Nizza 52, Torino, Italy. Istituto Nazionale di Fisica Nucleare (INFN), Sezione di Torino, Via Pietro Giuria 1, 10125 Torino, Italy.

[#]Corresponding author: Roberto Mazzoli,

Department of Life Sciences and Systems Biology. University of Turin. Via Accademia Albertina 13. 10123 Torino. Italy.

Tel. +39 011 6704644

Fax +39 011 6704508

E-mail: roberto.mazzoli@unito.it

Key words: ATP, acetate, ethanol, Alcohol dehydrogenase, pyruvate phosphate dikinase, glucose

Running title: Cellulose metabolism in *C. cellulovorans*

Abbreviations:

ABC	ATP binding cassette
ACAT	Acetyl-CoA acetyltransferase
ACK	Acetate kinase
ADH	Alcohol dehydrogenase
BCD	Butyryl-CoA dehydrogenase
BUK	Butyrate kinase
CAZY	Carbohydrate active enzyme
CBP	Consolidated bioprocesing
CoA	Coenzyme A

COGs	Cluster of Orthologous Genes
DSMZ	German Collection of Microorganisms and Cell Cultures
DTT	Dithiothreitol
ECH	Enoyl-CoA hydratase
F1,6BP	Fructose 1,6-bisphosphate
F6P	Fructose-6-phosphate
FDR	False discovery rate
GDH	Glutamate dehydrogenase
GlnS	Glutamine synthase
GluS	Glutamate synthase
HBD	Hydroxybutyryl-CoA dehydrogenase
MDH	Malate dehydrogenase
ME	Malic enzyme
PA	Pyruvic acid

PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate carboxylase
PEPCK	Phosphoenolpyruvate carboxykinase
PEPS	Phosphoenolpyruvate synthetase
PFL	Pyruvate formate lyase
PFOR	Pyruvate ferredoxin oxidoreductase
PK	Pyruvate kinase
PP_i	Pyrophosphate
PPDK	Pyruvate phosphate dikinase
PTA	Phosphate acetyltransferase
PTB	Phosphate butyryltransferase
PTS	Phosphotransferase system
SWATH-MS	Sequential Window Acquisition of All Theoretical Mass Spectra
TCA	Tricarboxylic acid

30

Abstract

Clostridium cellulovorans is among the most promising candidates for consolidated bioprocessing (CBP) of cellulosic biomass to liquid biofuels (ethanol, butanol). *C. cellulovorans* can metabolize all the main plant polysaccharides and its main catabolite is butyrate. This makes this strain a potential butanol producer since most reactions of butyrate and butanol biosynthesis from acetyl-CoA are common. Recent studies demonstrated that introduction of a single heterologous alcohol/aldehyde dehydrogenase diverts the branching-point intermediate, i.e. butyryl-CoA, towards butanol production in this strain. Despite *C. cellulovorans* potential for CBP of plant biomass, engineering its metabolic pathways towards industrial utilization requires better understanding of its metabolism. The present study aimed at improving comprehension of cellulose metabolism in *C. cellulovorans* by comparing growth kinetics, substrate consumption/product accumulation and whole-cell soluble proteome (data available via ProteomeXchange, identifier PXD015487) with those of the same strain grown on a soluble carbohydrate, glucose, as the main carbon source. Modulations of the central carbon metabolism in response to different growth substrate were detected, including regulation of glycolytic enzymes, fermentation pathways and nitrogen assimilation possibly affecting the redox balance. Higher energy expenditure seems to occur in cellulose-grown *C. cellulovorans*, which induces up-regulation of ATP synthetic pathways, e.g. acetate production and ATP synthase.

Significance

C. cellulovorans can metabolize all the main plant polysaccharides (cellulose, hemicelluloses and pectins) and, unlike other well established cellulolytic microorganisms, can produce butyrate. *C. cellulovorans* is therefore among the most attractive candidates for direct fermentation of lignocellulose to high-value chemicals and, especially, n-butanol, i.e. one of the most promising liquid biofuels for the future. Recent studies aimed at engineering n-butanol production in *C. cellulovorans* represent milestones towards production of biofuels through one-step fermentation of lignocellulose but also

indicated that more detailed understanding of the *C. cellulovorans* central carbon metabolism is essential to refine metabolic engineering strategies towards improved n-butanol production in this strain. The present study helped identifying key genes associated with specific catabolic reactions and indicated modulations of central carbon metabolism (including redox and energy balance) associated with cellulose consumption. This information will be useful to determine key enzymes and possible metabolic bottlenecks to be addressed towards improved metabolic engineering of this strain.

Introduction

Current awareness of the effects that fossil fuel exploitation brings about on global warming and climate changes has prompted search for alternative energy sources with lower environmental footprint [1]. Bio-based processes, such as biorefineries, have been especially promoted because of their potential significant benefits to environmental, economic and societal sustainability issues [2]. Lignocellulosic biomass is the most abundant raw material on the Earth, hence, it is among the most promising feedstock for the so-called 2nd generation biorefineries. Unlike 1st generation biorefineries, these processes rely on non-food biomass, such as agricultural/land by-products (e.g. cereal straw, forest residues), municipal or industrial wastes (e.g. paper mill sludge) [3]. However, lignocellulose is very recalcitrant to biodegradation and its bioconversion currently requires physical/chemical or enzymatic pre-treatment to improve its accessibility to enzymes and fermenting microorganisms [4,5]. Moreover, multiple bioreactors in series are necessary for enzyme production and/or polysaccharide hydrolysis and/or fermentation of soluble sugar(s) [2]. Development of consolidated bioprocessing (CBP) of lignocellulose, that is single-pot fermentation to high-value chemicals, could significantly reduce current costs of lignocellulose fermentation [6,7]. Since natural microorganisms isolated so far do not possess the biochemical features enabling CBP, metabolic engineering has been employed to develop improved microbial strains [7–10]. Candidates for CBP include both cellulolytic microorganisms such as *Clostridium thermocellum* and *Clostridium cellulolyticum* [9] and high-value compound producing yeasts and bacteria (e.g. *Saccharomyces cerevisiae*, lactic acid bacteria) [7,8].

C. cellulovorans is a strict anaerobic, mesophilic bacterium [11] among the most interesting candidates for CBP of plant biomass. *C. cellulovorans* shows some advantageous metabolic features with respect to other well established cellulolytic microorganisms such as *C. thermocellum*, *C. cellulolyticum* or *Thermoanaerobacterium saccharolyticum*: i) it ferments a larger panel of substrates that include all the main plant polysaccharides, namely cellulose, hemicelluloses and pectins [12,13]; ii) it produces butyril-CoA, which is a key intermediate for n-butanol production [11]. n-butanol is

89 considered the most promising liquid biofuel for future use in transportation [14]. With respect to
90 ethanol, butanol has higher combustion energy and can be used in pure form in engines (while ethanol
91 must be blended with gasoline) [15].

92 However, so far, research on *C. cellulovorans* has been mainly focused on its enzyme system
93 for depolymerizing plant polysaccharides. The latter features dozens of carbohydrate active enzymes
94 (CAZys) including both glycosyl hydrolases, carbohydrate esterases and polysaccharide lyases [16].
95 Remarkably, the number of CAZys encoded by *C. cellulovorans* genome is 37% higher than that found
96 in *C. thermocellum* (one of the most efficient cellulolytic microorganisms isolated so far) [8]. Several
97 of these enzymes, that is those containing a dockerin domain, are physically associated to form huge
98 protein complexes called cellulosomes, which are tethered to the *C. cellulovorans* cell surface [17].
99 Several studies have shown that the expression of *C. cellulovorans* cellulosomal and non-cellulosomal
100 CAZYs is modulated by the available growth substrate(s) [18–21].

101 Recently, gene tools for the manipulation of *C. cellulovorans* have been developed [22–24].
102 This led to improvement of production of liquid biofuels, namely ethanol and n-butanol, in *C.*
103 *cellulovorans* by metabolic engineering [25,26]. These studies have been milestones towards n-butanol
104 production by CBP of plant biomass, using a single microorganism. However, the n-butanol titer
105 obtained by these investigations (i.e. 3.47 g/L) is insufficient for industrial application and further
106 optimization of these strains is necessary [25,27]. Improvements of gene systems for *C. cellulovorans*
107 manipulation are desirable to increase transformation efficiency and success rate of gene modification
108 attempts [24,27]. Moreover, detailed understanding of *C. cellulovorans* central carbon metabolism is
109 essential to identify key genes and possible metabolic bottlenecks that should be addressed by
110 metabolic engineering strategies. As far as we know, only two previous studies have focused on
111 intracellular proteins involved in the metabolism of cellulose or other plant polysaccharides in *C.*
112 *cellulovorans* [12,13] which seem insufficient for the aforementioned purposes.

In the present study, a comparative approach was applied to *C. cellulovorans* cells grown with either crystalline cellulose (i.e. avicel) or a soluble carbohydrate, namely glucose, as the main carbon source. Previous studies on other cellulolytic clostridia have shown that overall metabolism on cellulose (and other polysaccharides) may highly differ from that observed on soluble mono- /di-saccharides, because of different kinetics and energetics of complex versus simple carbohydrate metabolism [2]. To gauge the global effects caused by cellulose utilization, we analyzed *C. cellulovorans* growth kinetics and substrate consumption/metabolite accumulation in conjunction with label-free quantitative proteomics.

Materials and Methods

GROWTH CONDITIONS

Clostridium cellulovorans was grown anaerobically in the DSMZ medium 320 (https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium320.pdf), with some modifications. Trypticase peptone and rumen fluid were not supplemented because not required for growth [11]. Na_2CO_3 was replaced by PIPES [piperazine-N,N′-bis(2-ethanesulfonic acid)] (15.12 g/l) as pH buffering agent [11]. Media were purged with pure N_2 (instead of 80:20 N_2-CO_2 mixture) and sterilized by autoclaving (20 min 121°C). Either 5 g/l D-glucose or 10 g/l avicel® PH-101 microcrystalline cellulose (50 µm particle size; Sigma-Aldrich Inc., St. Louis, MO, USA) were supplemented as the main carbon source. Inocula were grown in glucose-supplemented medium until exponential growth phase and then transferred into 500 ml butyl-stoppered bottles containing glucose- or avicel-supplemented medium. Cultures were incubated at 37°C without agitation. For each growth condition three independent cultures were performed.

ESTIMATION OF BACTERIAL GROWTH

Microbial growth was monitored at regular time intervals though estimation of total protein content. For protein extraction, 15 ml of culture was collected by centrifugation (4000 xg, 4°C, 20 min) and washed twice with 0.9% (w/v) NaCl. The cell pellet was re-suspended in 1 ml of 0.2 M NaOH and incubated 10 min at 100°C. Protein samples were quantified by using the Bradford reagent (Sigma-Aldrich Inc., St. Louis, MO, USA) following the manufacturer's instruction. Bovine serum albumin was used as the standard.

SUBSTRATE CONSUMPTION DETERMINATION

Glucose concentration in cell free supernatants was measured hourly using the glucose oxidase-peroxidase kit (K-GLUC, gopod format, Megazyme International, Bray, Ireland), following the manufacturer's instructions.

To determinate cellulose consumption, the cellulose concentration was measured from cell pellet every three days. Briefly, after two washing steps with 0.9% (w/v) NaCl, the pellet was re-suspended in 67% (v/v) H_2SO_4 and incubated for one hour under stirring at room temperature, to promote cellulose hydrolysis. The total carbohydrate quantification was performed with the phenol-sulfuric acid method [28], using glucose for the standard curve.

END-PRODUCT AND INTRACELLULAR ATP QUANTIFICATION

Accumulation of acetic acid, lactic acid, formic acid and butyric acid in the growth medium was quantified by high-pressure liquid chromatography (HPLC; Agilent Technologies 1200 series), equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA), a Micro-guard (cation H; Bio-Rad, Hercules, CA, USA) and a UV-Vis detector set at 210 nm. The mobile phase was 5 mM H_2SO_4 at a flux of 0.5 ml/min and a temperature of 50°C.

Ethanol in the growth medium was quantified by the Ethanol assay kit (K-EtOH, Megazyme International, Bray, Ireland), following the manufacturer's instructions.

For quantification of total intracellular ATP content, 1 ml of culture was collected and centrifuged at 10000 xg for 10 min. Pellets were resuspended in 200 µl of PBS, lysed with CellTiter-Glo® One Solution (Promega Corporation, Madison, WI, USA), and ATP content was measured by following the manufacturer's instructions.

PROTEOMIC ANALYSIS

Cytosolic protein extraction

Biomass samples were harvested (4000 xg, 4°C, 20 min) 5 hours and 7 days after inoculum from glucose- and avicel-supplemented cultures, respectively, and washed twice with 0.9% (w/v) NaCl. Protein were extracted according to Munir *et al.*, 2015 [29]. Briefly, the pellets were resuspended in 6 ml of 2% (v/v) SDT-lysis buffer (2 mM Tris-HCl, 0.4% (w/v) SDS, pH 7.6), with 100 mM DTT. The samples were incubated 10 min at 95°C and centrifuged (4000 xg, 20 min). The supernatant was centrifuged again (10 min, 10000 xg) to discard unlysed cells and cell debris. Proteins were precipitated from supernatants by methanol-chloroform method [30]. The protein pellets were resuspended in 25 mM NH_4HCO_3 with 0.1% (w/v) SDS and the protein concentration was measured by the 2-D Quant kit (GE Healthcare, Chicago, IL, USA), following the manufacturer's instructions.

In-solution protein digestion

Prior to SWATH-MS (Sequential Window Acquisition of all Theoretical fragment ion spectra Mass Spectrometry) [31,32] analysis, proteins were digested with trypsin. Briefly, the samples were prepared to have 100 µg of proteins in 25 µl of 100 mM NH_4HCO_3. The proteins were reduced by adding 2.5 µl of 200 mM DTT and incubating them at 90°C for 20 min, and alkylated with 10 µl of 200 mM iodoacetamide for 1 h at room temperature in the dark. Excess of iodoacetamide was finally removed by 200 mM DTT. After dilution with 300 µl of water and 100 µl of NH_4HCO_3 to raise the pH to 7.5-8.0, 5 µg of trypsin (Sequence Grade, Promega Corporation, Madison, WI, USA) was added and

digestion was performed overnight at 37 °C. Trypsin activity was stopped by adding 2 µl of neat formic acid and digests were dried by Speed Vacuum [33]. The samples were desalted on the Discovery® DSC-18 solid phase extraction (SPE) 96-well Plate (25 mg/well) (Sigma-Aldrich Inc., St. Louis, MO, USA) and then analyzed as previously described [34].

SWATH-MS analysis

LC–MS/MS analyses were performed using a micro-LC Eksigent Technologies (Dublin, OH, USA) system with a stationary phase of a Halo Fused C18 column (0.5 × 100 mm, 2.7 µm; Eksigent Technologies, Dublin, OH, USA). The mobile phase was a mixture of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B), eluting at a flow-rate of 15.0 µl/min at an increasing concentration of solvent B from 2% to 40% in 30 min. The LC system was interfaced with a 5600+ TripleTOF system (SCIEX, Concord, Canada). Samples used to generate the SWATH-MS spectral library were subjected to the traditional data-dependent acquisition (DDA) and to cyclic data independent analysis (DIA) of the mass spectra, using a 25-Da window as reported elsewhere [35]. The MS data were acquired with Analyst TF 1.7 (SCIEX, Concord, Canada). Three instrumental replicates for each sample were subjected to the DIA analysis [36].

Protein data search

The MS files were searched using Protein Pilot v. 4.2 (SCIEX, Concord, Canada) with the following parameters: cysteine alkylation, digestion by trypsin, no special factors and False Discovery Rate at 1%; The files were search also with Mascot v. 2.4 (Matrix Science Inc., Boston, USA) using trypsin as enzyme, with 2 missed cleavages and a search tolerance of 50 ppm was specified for the peptide mass tolerance, and 0.1 Da for the MS/MS tolerance, charges of the peptides to search for were set to 2 +, 3 + and 4 +, and the search was set on monoisotopic mass. The instrument was set to ESI-QUAD-TOF and the following modifications were specified for the search: carbamidomethyl cysteines as fixed modification and oxidized methionine as variable modification. The UniProt/Swiss-Prot reviewed

209 database containing *C. cellulovorans* proteins (NCBI_Clostridium_cellulovorans743B, version
210 30102017, 4278 sequence entries) was used.

211 *Protein quantification*

212 The label-free quantification was performed by integrating the extracted ion chromatogram of all the
213 unique ions for a given peptide using PeakView 2.0 and MarkerView 1.2. (Sciex, Concord, ON,
214 Canada). SwathXtend was employed to build an integrated assay library, built with the DDA
215 acquisitions, using a protein FDR threshold of 1 %. Six peptides per protein and six transitions per
216 peptide were extracted from the SWATH files. Shared peptides were excluded as well as peptides with
217 modifications. Peptides with FDR lower than 1 % were exported in MarkerView for the t-test. The up-
218 and down-regulated proteins were selected using p-value < 0.05 and fold change > 1.5.

219 The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via
220 the PRIDE [37] partner repository (https://www.ebi.ac.uk/pride/archive/) with the dataset identifier
221 PXD015487.

222 *Protein classification and statistical analysis*

223 For each primary annotations of proteins, files were downloaded from the National Center for
224 Biotechnology Information resource
225 (ftp://ftp.ncbi.nlm.nih.gov/genomes/refseq/bacteria/Clostridium_cellulovorans/), the PATRIC Bacterial
226 Bioinformatics Resource Center (https://www.patricbrc.org) and the eggNOG database of orthologous
227 groups and functional annotation (http://eggnogdb.embl.de/#/app/home). The eggNOG resource
228 provides Clusters of Orthologous Groups (COGs) of proteins
229 (https://www.ncbi.nlm.nih.gov/pubmed/9381173), which represent a framework for functional protein
230 classification on the basis of accurately deciphered evolutionary relationships
231 (https://www.ncbi.nlm.nih.gov/pubmed/26582926). Protein-level annotations overall *C. cellulovorans*
232 proteins are detailed in the **Supplementary File 1**. Fisher's exact test and fold enrichment were used to

identify significantly overrepresented COG categories in the up- and down-regulated proteins compared to the overall quantified proteins. Multiple testing adjustment for p-values derived from the Fisher's exact test was carried out using the Benjamini-Hochberg method.

For predicting both signal peptides and subcellular localization of the proteins involved in lignocellulose depolymerization Signal-P 4.1 (cut-off > 0.45) [38] (http://www.cbs.dtu.dk/services/SignalP-4.1/) and PSORTb v.3.0 [39] (https://www.psort.org/psortb/) were used, respectively.

Results and discussion

GROWTH CHARACTERISTICS AND FERMENTATION PRODUCTS

C. cellulovorans growth and substrate consumption in cultures with 5 g/l D-glucose and 10 g/l avicel (microcrystalline cellulose) are shown in **Fig. 1A, B**. Glucose supported around 16-fold higher growth rate (μ = 0.39 h^{-1}) with respect to cellulose (μ = 0.025 h^{-1}). These observations essentially confirm previous reports on *C. cellulovorans* [13,22]. The supplied substrates were only partially consumed, that is 3.1 g/l of cellulose (in 28 days) and 1.4 g/l of glucose (in 9 hours) were consumed in avicel- and glucose-supplemented cultures, respectively. Therefore, factors other than carbon substrate depletion (*e.g.* metabolite accumulation, pH) determined growth arrest. Actually, complete consumption of glucose (about 10 g/l in about 4 days) and higher consumption of cellulose (about 7 g/l in 10 days) by *C. cellulovorans* was previously reported by maintaining the pH of the culture between 6.0 and 7.0 [22] while no pH regulation was used in the present study.

All the main fermentation products, that is acetic acid, butyric acid, ethanol, formic acid and lactic acid (**Fig. 1C**, **Supplementary Figure 1**) were produced at higher yield in glucose-grown cultures. The sum of their final concentrations indicates that about 98% of the carbon derived from glucose was converted into these products. However, only 27% of the cellulose consumed by avicel-

grown *C. cellulovorans* was converted into these catabolites. Since the final bacterial biomass was similar in the two growth conditions studied, the rest of consumed cellulose should have taken other metabolic fates. Other cellulolytic bacteria, such as *Ruminiclostridium cellulolyticum* (previously *Clostridium cellulolyticum*) and *C. thermocellum*, may accumulate glycogen and/or exopolysaccharides and/or extracellular cellodextrins and/or amino acids [40,41]. This has never been reported in *C. cellulovorans*, but it could not be excluded, because of the scarce number of available studies on the metabolism of this strain. Further investigations necessary to test this hypothesis were beyond the scope of the present study. Butyric, formic and lactic acid were accumulated in similar amounts in the two growth conditions tested (**Fig. 1C**). Butyric and formic acid were the most abundant end-products while low amounts of lactic acid were detected. However, cellulose- and glucose-grown *C. cellulovorans* produced different amounts of acetate and ethanol (**Fig. 1C**). More in detail, cellulose-grown cultures accumulated higher amounts of acetate (t-test, p-value = 0.01) and lower amounts of ethanol (t-test, p-value = 0.02). Previous investigations reported no ethanol accumulation by glucose- or cellulose-grown *C. cellulovorans* [22,25]. However, growth media with significantly different composition and different pH regulation of bacterial cultures in these studies may justify the different catabolite profiles observed. Changes in metabolite production profiles between different growth conditions have been reported for other cellulolytic bacteria. In *Clostridium termitidis* CT1112, reduction in ethanol (and formate) production and increase in acetate accumulation was observed when it was grown on cellulose instead of a soluble sugar (*i.e.* cellobiose) [42]. In *R. cellulolyticum*, the carbon flux partition between the main catabolic products (i.e. acetate, ethanol and lactate) is greatly affected by pH and entering carbon flows, with higher acetate production from substrates which are more slowly metabolized [43]. Lower acetate production and higher ethanol accumulation as the growth rate increases has also been observed in *C. thermocellum* [44]. The results obtained in the present study are therefore consistent with previous observations on other cellulolytic clostridia. Interestingly, this study shows that cellulose promotes a pathway, i.e. acetate production, involved in

ATP synthesis, while it down-regulates ethanol production which is involved in NAD(P)H-consumption in *C. cellulovorans*.

COMPARATIVE PROTEOMIC STUDY

Overall findings

The present study was focused on soluble whole-cell extracts in order to identify proteins specifically associated with glucose or crystalline cellulose metabolism. Most previous proteomic studies on cellulose metabolism by *C. cellulovorans* were interested in extracellular proteins [19–21,42]. Only very recently, whole-cell proteomes of *C. cellulovorans* grown on either glucose- or cellulose-supplemented medium were compared for a total of 1016 identified proteins in both conditions [13]. Samples for the present investigation were harvested in the late exponential phase (**Fig. 1A, B)**. Quantitative proteomic analysis was performed by the data independent acquisition-based Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS) approach [31,32] combining deep proteome coverage capabilities with quantitative consistency and accuracy. In this study, 621 proteins were quantified (**Supplementary File 1**) corresponding to about 15 % of the *C. cellulovorans* annotated proteins [16]. To analyze the distribution of their biological functions, the quantified proteins were annotated by means of the Cluster of Orthologous Genes (COGs) categories (http://eggnogdb.embl.de/#/app/home). The large majority (522 proteins, 84 %) of quantified proteins were associated with at least a known function grasped by a COG category whereas the remaining 16 % consisted of proteins with unknown function (**Fig. 2A**). Of the total quantified proteins, 319 were found as differentially expressed when comparing the two growth conditions, that is they were at least 1.5-fold more abundant (p-value < 0.05) in one growth condition compared to the other.

Most (258 proteins, 81 %) differentially expressed proteins were classified into the variety of COG categories shown in **Fig. 2A** while the remaining 19% are still functionally uncharacterized. This suggests that proteins among those with yet unknown biological function could be involved in

cellulose/glucose metabolism thus confirming previous reports [19]. We noticed that 124 proteins corresponding to 39 % of the differentially expressed proteins were annotated as having a transport function. Actually, differentially expressed proteins were not fairly distributed across the COG categories (**Fig. 2B**). The proteins involved in cell duplication and protein translation were the largest COG categories shared by the proteins resulting down-regulated in cellulose-grown cells. This latter observation is likely related to the higher growth rate shown by glucose-grown cells. The proteins involved in carbohydrate transport and metabolism were instead the largest COG category in up-regulated proteins in cellulose-grown cultures. The latter observation was corroborated by the analysis of the functional enrichment with respect of all quantified proteins (Fisher's exact test, FDR < 0.05) whereby overexpressed proteins in cellulose-grown cultures were functionally enriched in the category of metabolism and transport of carbohydrates (**Fig. 2C**) and especially (74% of them) in plant polysaccharide depolymerization. In the following sections, differentially expressed proteins will be thoroughly discussed according to their functional classification.

Proteins involved in plant polysaccharide depolymerization

Many proteins that are more abundant in avicel-grown cultures are involved in plant polysaccharide depolymerization (**Table 1**). They include 24 out of 57 cellulosomal subunits encoded by the *C. cellulovorans* genome [45]. Cellulosomal subunits represent 24 out of 39 (62%) over-expressed proteins belonging to the class of carbohydrate transport and metabolism. Exoglucanase S (ExgS, Clocel_2823), *i.e*. a cellulase, Mannanase A (ManA, Clocel_2818), *i.e.* a hemicellulase, and CbpA (Clocel_2824), *i.e.* the main scaffolding protein of the *C. cellulovorans* cellulosome, showed the largest differential expression, since they were 44-fold, 36-fold and 33-fold more abundant in cellulose-grown bacteria, respectively. These proteins, together with additional up-regulated proteins identified in the current study (i.e., EngH, Clocel_2822; EngK, Clocel_2821; EngL, Clocel_2819; EngM, Clocel_2816; HbpA, Clocel_2820), are encoded by a large cellulosomal gene cluster on the *C. cellulovorans* chromosome [45]. Endoglucanase Z (EngZ, Clocel_ 2741) and Endoglucanase E

(Clocel_2576) were also among the most strongly up-regulated (19- and 17-fold, respectively) proteins, consistently with their key role in hydrolysis of crystalline cellulose [46] and for the function and architecture of the *C. cellulovorans* cellulosome [47,48], respectively.

Over-expressed non-cellulosomal proteins include three cellulases (EngD, Clocel_3242; EngO, Clocel_1478; Clocel_2606) and two hemicellulases (β-mannanase, Clocel_1134; β-xylosidase, Clocel_2595).

The present analysis showed that hemicellulases were expressed in cellulose-grown cells and the same cellulases and hemicellulases up-regulated in cellulose-grown cells were detected also in glucose-grown cells, although at a lower abundance. These data agree with most results obtained by previous transcriptomic/proteomic studies on *C. cellulovorans* which reported that several cellulosome components (e.g. CbpA, EngE, EngL, EngY, ExgS, ManA, and the products of genes Clocel_0619, Clocel_2575, Clocel_2576, Clocel_2607, Clocel_4119), including hemicellulases, are constitutively biosynthesized [19,49]. On the other hand, overexpression of some of these proteins (e.g. CbpA, EngB, EngE, and EngZ) is induced by crystalline cellulose [46,49].

Proteins involved in protein secretion

Three components of the Sec protein-translocation complex (SecF, Clocel_2074; SecD, Clocel_2075; SecY, Clocel_3713) were shown to be more abundant (1.9-, 1.8- and 1.7-fold, respectively) in cellulose-grown cells. This finding could be related to increased need to secrete CAZYs involved in cellulose depolymerization. Almost no information is available on mechanisms of secretion of cellulases in native cellulolytic microorganisms. For most (91 %) cellulases reported in UniProtKB (https://www.uniprot.org/help/uniprotkb) no signal peptide is annotated [50,51]. Among cellulases from Gram-positive bacteria, 10 % contained twin-arginine translocation (Tat)-like signal peptides, while 11 % featured amino acid patterns ascribable to the Sec secretory system [50,51]. The main components of the Sec system are a protein-conducting channel SecYEG, and an ATP-dependent motor protein SecA [52]. The auxiliary SecDF membrane protein-complex seems to enhance

translocation efficiency in a proton motive force-powered manner [52]. A signal peptide was predicted at the N-terminus of all the over-expressed proteins involved in cellulose/hemicellulose depolymerization identified in this study by Signal-P 4.1 (http://www.cbs.dtu.dk/services/SignalP-4.1/), except for Clocel_2595. More in detail, the N-terminus of 72 % of these proteins shows the typical structure of Sec-type signal peptides, that is a positively charged N-terminal (N-region), a hydrophobic core (H-region) and a negative charged C-region with alanine-rich cleavage site [53]. The variability occurring at the C-terminus of the cleavage site, with 67% of proteins displaying a VXA motif instead of the most typical AXA motif of the Gram-positive bacteria (**Fig. 3**) [51], may indicate that a different consensus sequence is used by *C. cellulovorans* for secreting many of its cellulases. Future investigations will be necessary to confirm this hypothesis and the possible role of the Sec machinery proteins (SecD, Clocel_2074; SecF, Clocel_2075; SecY, Clocel_3713) that were over-expressed in this study in cellulase secretion in *C. cellulovorans*. Understanding cellulase secretion in native cellulolytic microorganisms is crucial for improving comprehension of plant polysaccharide degradation and its exploitation in biotechnological applications such as the construction of recombinant cellulolytic microorganisms, which are frequently hampered by inefficient expression of heterologous cellulases [7].

Substrate uptake

It is generally considered that most cellulose is extracellularly converted to different length cellodextrins (rather than glucose) by anaerobic cellulolytic bacteria [40]. Cellodextrins are then transported into the cytoplasm and depolymerized mainly through phosphorolysis [40]. Seven proteins related to ATP binding cassette-type (ABC) transporters (Clocel_0040; Clocel_1357; Clocel_4050; Clocel_3636; Clocel_3461; Clocel_3460; Clocel_3857) were more abundant in cellulose-grown cells. Protein sequence alignments by the protein BLAST(https://www.uniprot.org/blast/) highlighted sequence identity ranging from 54 % to 86 % with proteins involved in the transport of a variety of

substrates in other clostridia. In addition, four soluble-binding proteins (Clocel_0038; Clocel_3201; Clocel_0435; Clocel_1358), that is proteins which present soluble substrates to the transport channel, were overexpressed in cellulose-grown cultures [54]. Amino acid sequence analysis of these proteins strongly indicated that some of them could be involved in the uptake of mono-/oligo-saccharides derived from extracellular cellulose depolymerization. More in detail Clocel_0040 (5-fold over-expressed in cellulose-grown cells) featured 64 % amino acid sequence identity with a carbohydrate ABC transporter membrane protein from *Clostridium sp.* DMS 8431 (SAMN04487886_10195). Similarly, the ABC transporter encoded by Clocel_3857 (1.6-fold more abundant in cellulose-grown cells) has 72 % sequence identity with the sugar ABC transporter ATP-binding protein of *Clostridium pasteurianum* (C1I91_02605). However, the proteins encoded by Clocel_1357, Clocel_3460 and Clocel_3461 are likely involved in other functions since they show higher sequence identity (50-70 %) with Clostridial peptide- and sodium-transport systems. The protein products of Clocel_4050 and Clocel_3636 lacked conserved residues required for the propagation of feature annotation. Furthermore, a modulation of the expression of sugar phosphotransferase systems (PTS) was observed. PTS are multicomponent transporters that couple sugar transport with phosphorylation using phosphoenolpyruvate (PEP) as energy source [55]. Our analysis showed that some PTS components, namely the phosphotransferase system lactose/cellobiose-specific IIB subunit (Clocel_2881) and the permease IIC component (Clocel_2880), were overexpressed while other subunits, such as the phosphoryl carrier protein (Clocel_2058) and the phosphoenolpyruvate-protein phosphotransferase (Clocel_3686), were down-regulated in cellulose-grown cultures. A cytoplasmic cellodextrin phosphorylase (Clocel_2717) was also identified in this study, but its expression level did not significantly change between the two growth conditions considered.

Central carbon metabolism

Glycolysis

C. cellulovorans metabolizes glucose through the Embden-Meyerhof-Parnas pathway [16] (**Fig. 4A**). Our analysis revealed that the expression of several *C. cellulovorans* glycolytic enzymes was affected by carbon source change, albeit at different extent (**Fig. 4A**, **B)**. Phosphofructokinases generally control glycolysis and are allosterically activated by ADP and inhibited by fructose-6-P (F6P) and ATP in Clostridia [13,56]. The *C. cellulovorans* genome encodes two ATP-dependent 6-phosphofructokinases (namely Clocel_2901 and Clocel_0388), *i.e*. enzymes that catalyze the phosphorylation of F6P to fructose 1,6-bisphosphate (F1,6BP) by consuming ATP. The present study revealed that Clocel_0388 was not differentially expressed, whereas Clocel_2901 was 5-fold more abundant in cellulose-grown cells. Overexpression of Clocel_2901 in cellulose-grown *C. cellulovorans* (fold change, FC, comprised between 4 and 16) was also reported by another recent study [13]. *C. cellulovorans* genome also encodes a pyrophosphate (PP_i)-fructose 6-phosphate 1-phosphotransferase (Clocel_1603) (that catalyzes the phosphorylation of F6P using PP_i) but this enzyme was not found as differentially expressed in the current study. This observation could indicate a preferential ATP-dependent conversion of F6P in the cells grown on cellulose. Furthermore, four glycolytic enzymes were present in lower abundance in cellulose-grown cells: triosephosphate isomerase (Clocel_0721, FC = 0.03), glyceraldehyde-3-phosphate dehydrogenase (Clocel_0719, FC = 0.23), glucose-6-phosphate isomerase (Clocel_1364, FC = 0.25), and phosphoglycerate kinase (Clocel_0720, FC = 0.6). In general, these results do not seem to differ from those previously reported by Aburaya et al. [13], since most of these enzymes were slightly down-regulated in cellulose- versus glucose grown *C. cellulovorans* (with the only exception of the late stationary phase). Glyceraldehyde-3-phosphate dehydrogenase is among the most important glycolytic enzymes since it catalyzes NADH production through oxidation of glyceraldehyde-3-phosphate and has previously been identified as a probable bottleneck in glycolysis [57] and towards alcoholic biofuel production [58]. Glyceraldehyde-3-phosphate dehydrogenase up-regulation in glucose-grown *C. cellulovorans* agrees with previous studies on *C. termitidis* [42] and *C. thermocellum* [59], that reported that the same enzyme was one of the most abundantly expressed proteins during growth on cellobiose, a soluble β-glucose disaccharide.

435

436 *Pyruvate metabolism*

437 Interconversion between pyruvate (PA) and phosphoenolpyruvate (PEP) is most frequently mediated
438 by the antagonistic enzymatic couple consisting of ADP-dependent pyruvate kinase (PK) (catabolic
439 role) and phosphoenolpyruvate synthetase (PEPS) (anabolic role). In *C. cellulovorans* PK is encoded
440 by Clocel_0389 whereas no PEPS is annotated. However, *C. cellulovorans* genome encodes a pyruvate
441 phosphate dikinase (PPDK, Clocel_1454) that may catalyze the reversible production of PA, ATP and
442 P_i from PEP, AMP and PP_i [60]. In some organisms such as *Acetobacter xylinum*, *Propionibacterium*
443 *shermanii* and *Microbispora rosea* [61–63], PPDK seems to exert its activity in the anabolic (PEP)
444 direction [64,65], whereas it fulfils a catabolic role in others, such as *Clostridium symbiosum* [66]. In
445 *Thermoproteus tenax*, the combined action of PPDK, PK and PEPS was shown to control the
446 interconversion between PEP and PA [67]. In *C. thermocellum*, which does not possess PK, PPDK can
447 substantially support the production of PA from PEP [68]. In the present study, PK did not show any
448 differential expression between the two growth conditions, while PPDK showed 11-fold higher
449 abundance in cellulose-grown cells. Consistently, a 3.5-fold up-regulation was observed also for a
450 PPDK putative regulatory protein (Clocel_4349) [69]. Up-regulation of PPDK (Cter_0809) in
451 cellulose-grown cells was reported also in *Clostridium termitidis CT1112* [42]. Previous
452 characterization of PK and PPDK from different microorganisms has shown that their activity is
453 generally allosterically regulated. More in details, PPDK is regulated by the ATP/AMP ratio in
454 *Trypanosoma brucei* and *Acetobacter xylinum* [70,71] whereas PK activity by intracellular
455 concentration of ADP, AMP and phosphate sugars [72–74]. The present study points at PPDK as a
456 possible key enzyme regulating carbon flux during cellulose metabolism by *C. cellulovorans*. Further
457 investigations on this enzyme, such as understanding potential regulation by allosteric effectors, appear
458 essential to better understand its role in *C. cellulovorans* metabolism.
459 In some bacteria, malic enzyme (ME), malate dehydrogenase (MDH) and phosphoenolpyruvate
460 carboxykinase (PEPCK) are involved in an alternative three-step pathway converting PEP to pyruvate

that is called malate shunt [75] (**Fig. 4A**). In *C. thermocellum*, the malate shunt was proposed as a strategy to transfer electrons from NADH to $NADP^+$, thus supplying most of the NADPH necessary for biosynthetic routes [68]. A putative ME (Clocel_0393) was down-expressed, *i.e.* 3-fold less abundant, in cellulose-grown *C. cellulovorans*. We were therefore interested to understand if a malate shunt could be present in *C. cellulovorans* also. In its genome, two lactate/malate dehydrogenase are annotated (Clocel_1533; Clocel_2700). The protein product of Clocel_1533 shares an amino acid sequence identity of 40% with *C. thermocellum* MDH (Cthe_0345) which includes key residues in the active site [76]. Although a confirmation by enzyme activity assay is necessary, these findings strongly suggest that Clocel_1533 encodes a MDH. No PEP carboxykinase is annotated in the *C. cellulovorans* genome, but PEP carboxylase (PEPC, Clocel_1149), that catalyzes the conversion of PEP to oxaloacetate [77], could functionally replace it. PEPC was identified in the present study, although in similar amounts in the two growth conditions. Hence, a malate shunt could be hypothesized in *C. cellulovorans*, especially to supply NADPH for biosynthetic reactions in fast growth conditions such as in glucose-supplemented cultures. Further investigations by enzyme activity assay of the products of Clocel_1533 and Clocel_1149 will be necessary to confirm this hypothesis. Since previous studies reported that ME is allosterically inhibited by PP_i [78] and PPDK activity controls the intracellular PP_i concentration, a possible regulatory interconnection between the two pathways could exist.

Tricarboxylic acid cycle and nitrogen assimilation

C. cellulovorans can partially operate the tricarboxylic acid (TCA) cycle in a reductive manner [77]. Clostridia use TCA cycle mainly to produce intermediates for biosynthetic routes and regulate the redox balance inside the cells. According to the present study, biosynthetic levels of aconitase (Clocel_1405), citrate synthase (Clocel_3688), isocitrate dehydrogenase (Clocel_2469) and fumarate hydratase (Clocel_0392) were not affected by the carbon sources used. However, glutamate dehydrogenase (GDH, Clocel_1284) was 10-fold more abundant in glucose-grown bacteria. Furthermore, glutamine synthetase (GlnS, Clocel_3873) and glutamate synthase (GluS, Clocel_2665)

were found as 3- and 1.5-fold more abundant in glucose- versus cellulose-grown *C. cellulovorans*, respectively. All these proteins are typically involved in nitrogen assimilation and synthesis of components of cell biomass in bacteria. GDH catalyzes the reversible NAD(P)H-dependent reductive amination of 2-ketoglutarate to glutamate [79]; GlnS aminates glutamate to glutamine in ATP-dependent manner; GluS catalyzes the reversible transfer of an amino group from glutamine to 2-ketoglutarate with the consumption of NAD(P)H (or reduced ferredoxin) and the production of 2 glutamate molecules and $NAD(P)^+$ (or oxidized ferredoxin) [41]. In addition, intracellular accumulation of glutamate and secretion of amino acids (up to 15-17 % of the total substrate consumed) have been reported in other cellulolytic clostridia (e.g. *C. thermocellum* and *R. cellulolyticum*) [41,80]. However, overexpression of GDH in *C. thermocellum* was reported for cells growing on cellulose with respect to cultures growing on a soluble carbohydrate (*i.e.* cellobiose) [81]. Higher abundance of these enzymes in glucose-grown *C. cellulovorans* could be related with higher growth rates measured in this condition. In addition, activities of these proteins affect the redox balance of the cell. A recent study on *C. thermocellum* demonstrated that deletion of the gene *glnA* encoding its main glutamine synthetase significantly decreases amino acid secretion and increases ethanol yield likely by increasing NADH availability in the cell [41]. Finally, GDH, GlnS and GluS have been referred as involved in pH homeostasis in *C. thermocellum* [80]. However, no significant difference in extracellular pH between glucose- and cellulose grown *C. cellulovorans* cultures has been detected in the present study (data not shown).

End-product synthesis pathways

The main products of *C. cellulovorans* metabolism are H_2, CO_2, acetic acid, lactic acid, butyric acid and ethanol [11]. The proteins encoded by three out of the four hydrogenase genes annotated in the *C. cellulovorans* genome [82] were not identified in this study while the Fe-only hydrogenase (Clocel_4097) resulted 1.5-fold more abundant in glucose-grown cells. Therefore, the protein encoded by Clocel_4097 may be the main responsible for H_2 production in this strain. Hydrogen production is

513 the fastest way to dispose of excess of reduced cofactors generated through carbohydrate metabolism
514 [83]. Up-regulation of Clocel_4097 in glucose-grown cells, appears as a strategy to maximize
515 glycolytic turnover in this condition. This Fe-only hydrogenase probably receives electrons from the
516 oxidative activity of pyruvate ferredoxin oxidoreductase PFOR (Clocel_1684), which converts
517 pyruvate to acetyl-CoA, yielding CO_2 and reduced ferredoxin (**Fig. 4A**) Consistently, PFOR was found
518 1.6-fold more abundant in glucose-grown cells. According to gene annotation, *C. cellulovorans*
519 genome encodes another PFOR (Clocel_2840) and an indole pyruvate oxidoreductase subunit
520 (Clocel_4184), that were not detected in the present study. These data therefore point at Clocel_1684 as
521 the main PFOR in *C. cellulovorans*.
522 Another enzyme that was more abundant (2-fold) in glucose-grown cells is pyruvate formate lyase
523 (PFL, Clocel_1811). This enzyme catalyzes pyruvate conversion to formate and acetyl-CoA production
524 from pyruvate (**Fig. 4A**). Consistently, a pyruvate-formate lyase activating enzyme (Clocel_1812) [84]
525 was also found as 5-fold more abundant in the same growth condition.
526 *C. cellulovorans* genome encodes seven alcohol dehydrogenases (ADHs), four of which were
527 overexpressed in cellulose-grown bacteria: Clocel_2402 (2.5-fold), Clocel_1990 (8-fold), Clocel_3817
528 (1.6-fold), Clocel_1140 (2.2-fold) (**Supplementary File 1**). The presence of multiple ADHs is
529 common in solvent-producing clostridia and other microorganisms [85,86]. ADHs can differ for their
530 substrate and coenzyme specificity, but the physiological significance of multiple ADHs in the same
531 strain has not always been elucidated. Clocel_2402, Clocel_3817 and Clocel_1140 encode bifunctional
532 aldehyde/alcohol dehydrogenases, possibly involved in 2-step reduction of acetyl-CoA to acetaldehyde
533 and finally to ethanol with consumption of two NADH (**Fig. 4A**). The amino acid sequence of ADH
534 encoded by Clocel_1990 shows 89% identity with glycerol dehydrogenases from other Clostridia, *i.e*.
535 enzymes that catalyze the oxidation of glycerol [87]. However, owing to the scarce substrate specificity
536 generally shown by ADHs, it is difficult to definitely assess their role in ethanol or other alcohol
537 production without enzyme activity assays. Up-regulation of these ADHs in cellulose-grown *C.*
538 *cellulovorans* is not reflected by increased ethanol production since a lower amount of ethanol is

accumulated in this growth condition (**Fig. 1C**). Apart from possible involvement of these up-regulated enzymes in other alcohol synthesizing pathways, a reasonable explanation for this observation is that ADHs may be reversible enzymes and also catalyze alcohol oxidation. Most studies on characterization of ADH from clostridia focused on their substrate and coenzyme specificity but few papers reported clostridial ADHs able to oxidize alcohols, although their activity was mainly in the aldehyde-reducing direction [88,89]. Previous studies showed that relative protein abundances do not always correlate with end-product distribution profiles. In particular, higher abundance of ADHs with respect to lactate dehydrogenase was observed in some growth conditions in *Thermoanaerobacter thermohydrosulfuricus* WC1, however, lactate was always the major end-product of this strain [90]. The present study confirms that protein abundance is not the only player determining carbon flux distribution in cells, which is also influenced by other parameters such as catabolic bottlenecks, allosteric regulation or cofactor availability.

Phosphate acetyltransferase (PTA, Clocel_1891), which catalyzes the conversion of acetyl-CoA to acetyl-phosphate, was found up-regulated (1.9-fold) in cellulose-grown cells whereas acetate kinase (ACK, Clocel_1892), which catalyzes the subsequent conversion of acetyl-phosphate to acetate and ATP did not result as differentially expressed in the present study (**Fig. 4B**). Genes encoding PTA and ACK form an operon in *C. cellulovorans*, so, our results indicate that post-transcriptional events may differentially regulate the expression of these genes. Changes in proteomic levels of PTA are consistent with increased production of acetate by cellulose-grown *C. cellulovorans* (**Fig. 1C**). These data also suggest that the level of PTA activity could be a bottleneck for acetate production in *C. cellulovorans*, while ACK could have a higher specific activity.

Butyric acid derives from acetyl-CoA through a multi-step pathway as shown in **Fig. 4A**. Some of the enzymes involved in this pathway are also implicated in other metabolic processes, such as fatty acid and amino acid metabolism. Maybe for this reason, some of them, that is acetyl-CoA acetyltransferase (ACAT, Clocel_3058), hydroxybutyryl-CoA dehydrogenase (HBD, Clocel_2972), enoyl-CoA hydratase (ECH, Clocel_2976) and phosphate butyryltransferase (PTB, Clocel_3675), were down-

565 regulated while butyryl-CoA dehydrogenase (BCD, Clocel_2975) and butyrate kinase (BUK,
566 Clocel_3674) were up-regulated in cellulose-grown cells. Actually, no significant changes in butyrate
567 amounts accumulated by cellulose- or glucose-grown *C. cellulovorans* was observed in this study.
568
569 **ENERGY PRODUCTION**

570 Three components of the F_0F_1-ATP synthase, i.e. ATP synthase F_1 sector subunit alpha (Clocel_3051)
571 and subunit beta (Clocel_3049) and F_0 portion subunit c (Clocel_3054), were 2-fold more abundant in
572 cellulose-grown *C. cellulovorans*. ATP synthase F_0 subunit b (Clocel_3053) and F_1 gamma chain
573 (Clocel_3050) did not show any differentially expression between the two growth conditions. The
574 genes encoding these proteins together with Clocel_3048, Clocel_3052 and Clocel_3055 form an
575 operon which codes for the entire ATP synthase complex [16]. Some F_0F_1-ATP synthases of strict
576 anaerobes mainly hydrolyze ATP to pump H^+ out of the cell, such as in *C. thermocellum* where this
577 function has been related to pH homeostasis [80,91,92]. However, no significant difference in
578 extracellular pH between glucose- and cellulose-grown *C. cellulovorans* cultures has been detected in
579 the present study (data not shown). Other strict anaerobic bacteria have ATP synthases which mainly
580 function towards ATP synthesis by using either Na^+, such as *Propionigenium modestum* [93] and
581 *Acetobacterium woodii* [94], or H^+, *e.g. Moorella thermoacetica* [95], transmembrane gradient as
582 energy source. In anaerobes, Na^+/H^+ gradient can be generated by several mechanisms such as
583 anaerobic electron chains, H_2 oxidation [91] and membrane bound proton-translocating
584 pyrophosphatases [59]. It can therefore be hypothesized that up-regulation of F_0F_1-ATP synthase could
585 provide additional ATP to that generated by substrate-level phosphorylation in *C. cellulovorans* (**Fig.**
586 **4A**).
587
588 **ATP QUANTIFICATION**

589 Metabolite and proteomic analyses indicated an up-regulation of acetate biosynthetic pathway in
590 cellulose-grown *C. cellulovorans*. Acetate, as well as butyrate, production is involved in ATP synthesis
591 through substrate-level phosphorylation. Furthermore, over-expression of some F_0F_1 ATP synthase
592 subunits was detected in cellulose-grown *C. cellulovorans*. These observations suggest possible
593 differences in ATP-content of glucose- or cellulose-grown cells. In most growth phases, the ATP
594 content of cellulose-grown bacteria ranged between 0 and 3 ng/mg, that is much lower (about a 100
595 times) than that measured in glucose-grown cells (**Fig. 5**). Interestingly, ATP content of cellulose-
596 grown cultures at the time of inoculum is of the same order of magnitude of that observed in glucose-
597 grown cells. It is worth reminding that both glucose- and cellulose-grown cultures were inoculated with
598 glucose-grown bacteria, hence, just after inoculum, *C. cellulovorans* cells likely still show most
599 characteristics associated with glucose metabolism. Our findings seem to contradict previous studies
600 claiming bioenergetic benefits for anaerobic bacteria grown on cellulose instead of simple
601 carbohydrates [40,96]. Increased acetate production and up-regulation of ATP synthase subunits by
602 cellulose-grown *C. cellulovorans* can be interpreted as a mean to improve ATP cell supply in
603 conditions requiring high ATP consumption (e.g. for cellulase biosynthesis and secretion) [97].
604

605 # Conclusions

606 The present comparative analysis indicated that the metabolism of cellulose-grown *C. cellulovorans*
607 significantly differs from that of the same strain grown on simple carbohydrates, i.e. glucose, thus
608 confirming previous reports on other cellulolytic microorganisms [40]. Apart from modulation of the
609 expression of cellulosomal and non-cellulosomal enzymes directly involved in cellulose
610 depolymerization, the present investigation revealed that other modifications of the metabolic network
611 are induced by the growth substrate. The up-regulation of some subunits of the Sec-machinery in
612 cellulose-grown *C. cellulovorans* point at them as interesting candidates possibly involved in cellulase
613 secretion in this strain. This could be a starting point for filling the gaps in understanding the

614 mechanisms of cellulase secretion in cellulolytic microorganisms. The most original findings brought
615 by the present study concern modifications of the central metabolism and fermentative pathways. It is
616 worth noting that previous studies did not report any significant modification in fermentation product
617 profile [22] or any major change in the expression of enzymes involved in the central metabolism
618 between glucose- and cellulose-grown *C. cellulovorans* [13]. This is most probably caused by different
619 composition [22] and/or different pH regulation of the growth medium [13]. In some cases, the present
620 study helped to identify the key genes associated with specific catabolic reactions or pathways among
621 multiple paralogs with the same annotation in *C. cellulovorans* based on their expression levels (e.g.
622 H_2ase, PFOR). In other cases (*e.g.* ADHs, MDH), our results clearly indicated that further studies (e.g.
623 substrate and cofactor specificity, catalytic activity regulation) are essential to understand the
624 physiological role of certain gene products. The main findings of the present investigation can be
625 summarized as follows:

626 • Proteomic data indicate that a re-distribution of the central carbon flow occurs between glucose-
627 and cellulose-grown *C. cellulovorans*, through a modulation of the biosynthesis of different key
628 enzymes that include ATP-dependent 6-phosphofructokinase (Clocel_2901), PPDK
629 (Clocel_1454), GDH (Clocel_1284), PTA (Clocel_1891), PFL (Clocel_1811) and different
630 ADHs.
631 • A macroscopic consequence of this re-arranged metabolic network is that cellulose promotes
632 acetate accumulation, while glucose induces higher ethanol production. Based on previous
633 reports, this could depend on the different substrate used, or the different growth rate/carbon
634 flux supported by these substrates [42–44].
635 • Cellulose-grown cells have significantly lower ATP content, possibly related to higher energy
636 expenditure for cellulase biosynthesis and secretion. Up-regulation of acetate pathway and ATP
637 synthase subunits may help cope with this high energy demanding condition. Therefore, these
638 observations seem to contradict previous studies claiming bioenergetic benefits of anaerobic
639 bacteria growing on cellulose instead of simple carbohydrates [40,96].

In conclusion, this study pointed out some aspects of glucose and cellulose metabolism in *C. cellulovorans*, which could be useful for better understanding the physiology of this strain and also towards engineering of its metabolic pathways for application in processes for biorefining plant biomass.

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Conflict of interest

The authors declare no conflict of interest

Author contributions

RM designed research and supervised experiments. GU, SC, AR and MM performed experiments and analyzed proteomic data. All the Authors contributed in discussing experimental data and writing the manuscript.

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936

Figure captions

Figure 1. *C. cellulovorans* growth kinetics in a medium containing 5 g/l glucose (**A**) or 10 g/l cellulose (**B**) as the main carbon source. Growth curves are represented by solid lines, while dashed lines represent substrate concentration. Bacterial biomass was measured by total cell protein determination. Syringe symbols indicate the cell sampling points for proteomic analysis, i.e. 5 hours and 7 days after inoculum for glucose- and cellulose-supplemented media, respectively. End-product final concentration (g/l) measured in glucose- (purple) or cellulose- (green) supplemented *C. cellulovorans* cultures (**C**). Data are displayed as mean ± SD across triplicate cultures. Asterisks indicate statistically significant differences (t-test, p-value ≤ 0.05) between the two growth conditions.

Figure 2. Functional categorization of quantified proteins. (**A**) Cluster of Orthologous Genes (COG) functional classification of the quantified proteins along with the differentially expressed proteins (DEP) resulting from the comparison between *C. cellulovorans* cultures grown on cellulose or glucose as the main carbon source. (**B**) COG-based functional classification of the proteins resulting up- or down-regulated when comparing *C. cellulovorans* cultures grown on cellulose or glucose as the main carbon source. (**C**) Functional enrichment analysis of up- and down-regulated proteins according to COG annotation. The dashed line corresponds to a fold enrichment equal to 1.5. Statistically significant over-representation of a COG category in the up- or down-regulated proteins (Fisher's exact test, FDR < 0.05) is labelled with an asterisk.

Figure 3. Consensus sequence of N-terminal signal peptide of the *C. cellulovorans* proteins involved in plant polysaccharide depolymerization that were up-regulated in cellulose-grown cultures.

Figure 4. (**A**) Scheme representing the metabolic changes observed in both *C. cellulovorans* central carbon metabolism and end-product synthetic pathways, based on protein differential expression. (**B**) Function, gene locus and fold change of the proteins involved in the central carbon metabolism and end-product synthesis in *C. cellulovorans*. Up- and down-regulated proteins are displayed in green and purple, respectively. Proteins that are not differentially expressed or not quantified in the present study are indicated in grey. GPI, glucose 6-phosphate isomerase; PFK, phosphofructokinase; ALD, aldolase; TPI, triosephosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK,

968 phosphoglycerate kinase; PMG, phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; PPDK,
969 pyruvate phosphate dikinase; PEPC, phosphoenolpyruvate carboxylase; PFOR, pyruvate ferredoxin
970 oxidoreductase; PFL, pyruvate formate lyase; ME, malic enzyme; MDH, malate dehydrogenase. Fd-
971 H2ase, ferredoxin-hydrogenase; ADH, bifunctional aldehyde/alcohol dehydrogenase; PTA, phosphate
972 acetyltransferase; ACK, acetate kinase; ACAT, acetyl-CoA acetyltransferase; HBD, hydroxybutyryl-
973 CoA dehydrogenase; ECH, enoyl-CoA hydratase; BCD, butyryl-CoA dehydrogenase; PTB, phosphate
974 butyryltransferase; BUK, butyrate kinase.

975

976 **Figure 5.** Intracellular ATP concentration measured in glucose- (**A**) and cellulose-grown *C.*
977 *cellulovorans* (**B**). Intracellular ATP concentration is expressed as Log_2 ng of ATP per mg of proteins
978 extracted from cell biomass. Data are displayed as mean ± SD of triplicate cultures.

979

980 **Supplementary File 1.** List of the quantified proteins in *C. cellulovorans* grown on avicel and glucose.
981 The fold-change is given by the ratio between the average of the protein abundances (based on
982 SWATH-MS data acquisition) for the three replicates of *C. cellulovorans* grown on avicel and the
983 average of the protein abundances (based on SWATH-MS data acquisition) of the three replicates in *C.*
984 *cellulovorans* grown on glucose. The proteins with fold-change > 1.5 and p-value > 0.05 are considered
985 as up-regulated (green), while the proteins with fold-change < 0.67 and p-value > 0.05 are considered
986 down-regulated (purple).

987

988 **Supplementary Figure 1**. Kinetics of accumulation of end-product measured in glucose- (**A**) or
989 cellulose- (**B**) supplemented *C. cellulovorans* cultures. Data are displayed as mean ± SD across
990 triplicate cultures.

Table I

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Table 1. Up-regulated proteins involved in plant polysaccharide depolymerization. Protein function, gene locus, CAZy category, fold change, signal peptide prediction (Signal-P 4.1, D-value > 0.5) and cellular localization prediction (PsortB v3.0) are shown. GH, glycoside hydrolase; CBM, carbohydrate binding module; PL, pectate lyase; SLH, S-layer homology; NA, not annotated.

Function	Gene locus	CAZy	FC	Signal-P	Psortb
Cellulosomal proteins					
Exoglucanase S	Clocel_2823	GH48	43.62	0.88	Extracell
Mannanase A	Clocel_2818	GH5	35.86	0.78	Unknown
Cellulososse-binding protein A	Clocel_2824	CBM3, SLH, HBD	33.49	0.89	Extracell
Endoglucanase	Clocel_2576	GH9, CBM3	19.41	0.74	Extracell
Endoglucanase Z	Clocel_2741	GH9, CBM3	18.64	0.55	Extracell
Dockerin type 1	Clocel_3193	NA	17.38	0.57	Extracell
Endoglucanase E	Clocel_3359	GH5, CBM65, SLH	16.93	0.82	Unknown
Endoglucanase L	Clocel_2819	GH9	14.49	0.80	Membrane
β-xylanase	Clocel_2900	GH10, CBM22	11.29	0.80	Extracell
Endoglucanase H	Clocel_2822	GH9, CBM3	10.97	0.57	Extracell
Mannanase	Clocel_4119	GH26, CBM35	9.83	0.73	Unknown
Endoglucanase	Clocel_0983	GH5	8.48	0.85	Unknown
Mannanase	Clocel_2575	GH26, CBM35	7.81	0.61	Unknown
Mannanase	Clocel_2607	GH26, CBM35	6.83	0.84	Extracell
Endoglucanase Y	Clocel_1624	GH9, CBM30	6.71	0.75	Extracell
Endoglucanase	Clocel_2600	GH5, CBM32	5.97	0.78	Extracell
Endoglucanase M	Clocel_2816	GH9, CBM4	5.94	0.54	Extracell
Endoglucanase B	Clocel_1150	GH5	5.03	0.68	Membrane
Endoglucanase	Clocel_3111	GH5	5.00	0.61	Extracell
Hydrophobic protein A	Clocel_2820	SLH, HBD	4.97	0.66	Extracell
Endoglucanase	Clocel_0619	GH5	4.69	0.86	Extracell
Endoglucanase	Clocel_0930	GH9, CBM3	4.50	0.78	Extracell
Endoglucanase K	Clocel_2821	GH9, CBM4	4.38	0.80	Extracell
Pectate lyase A	Clocel_1623	PL1, PL9	4.35	0.57	Extracell
Non-cellulosomal proteins					
Endoglucanase O	Clocel_1478	GH9, CBM4	6.06	0.60	Extracell
B-mannanase	Clocel_1134	GH26, CBM23	4.22	0.66	Cell wall
Endoglucanase D	Clocel_3242	GH5, CBM2	3.35	0.59	Membrane
β-xylosidase	Clocel_2595	GH43	3.16	-	Unknown
Endoglucanase	Clocel_2606	GH5, CBM46	2.64	0.64	Cell wall

Figure 1

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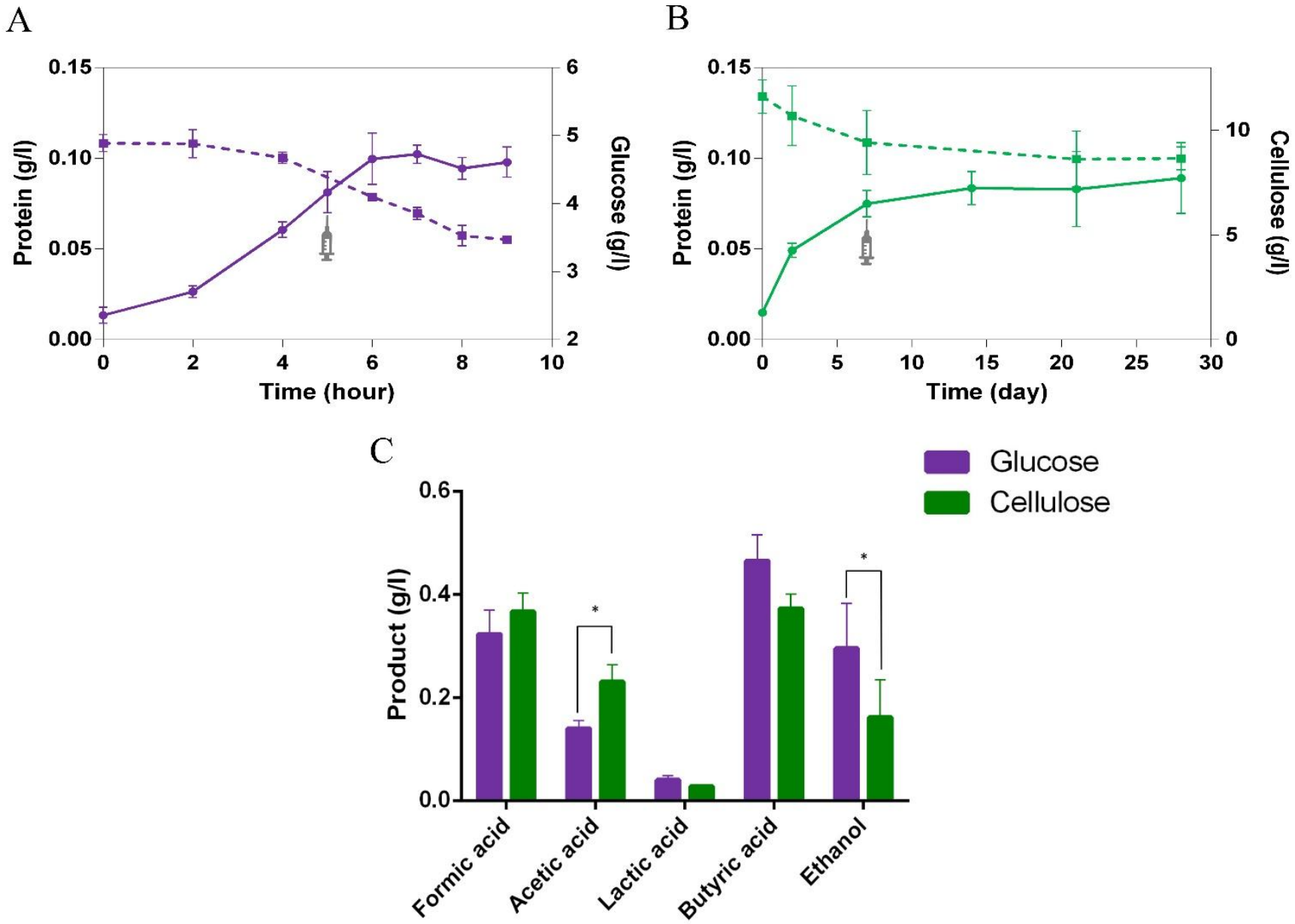
Figure 1



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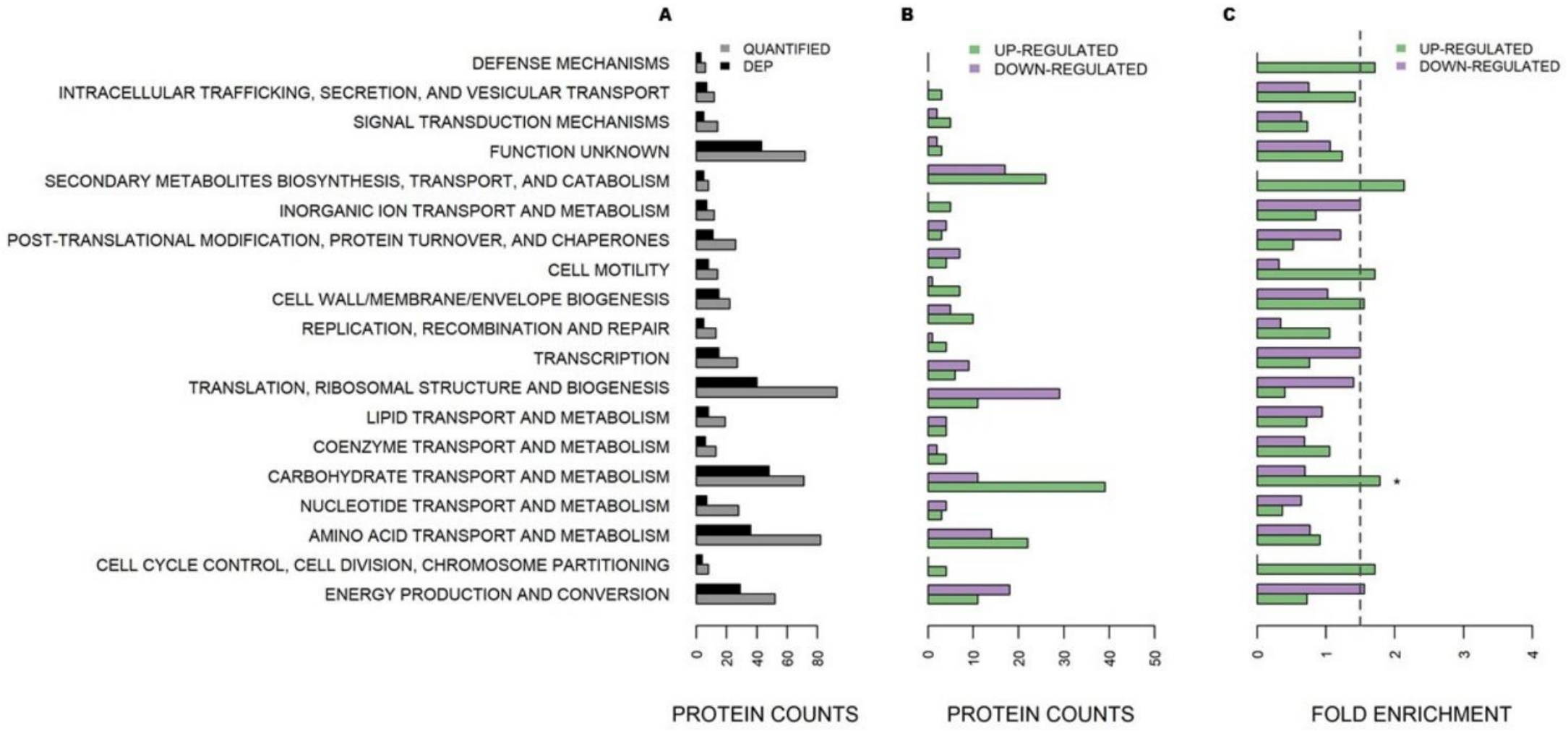

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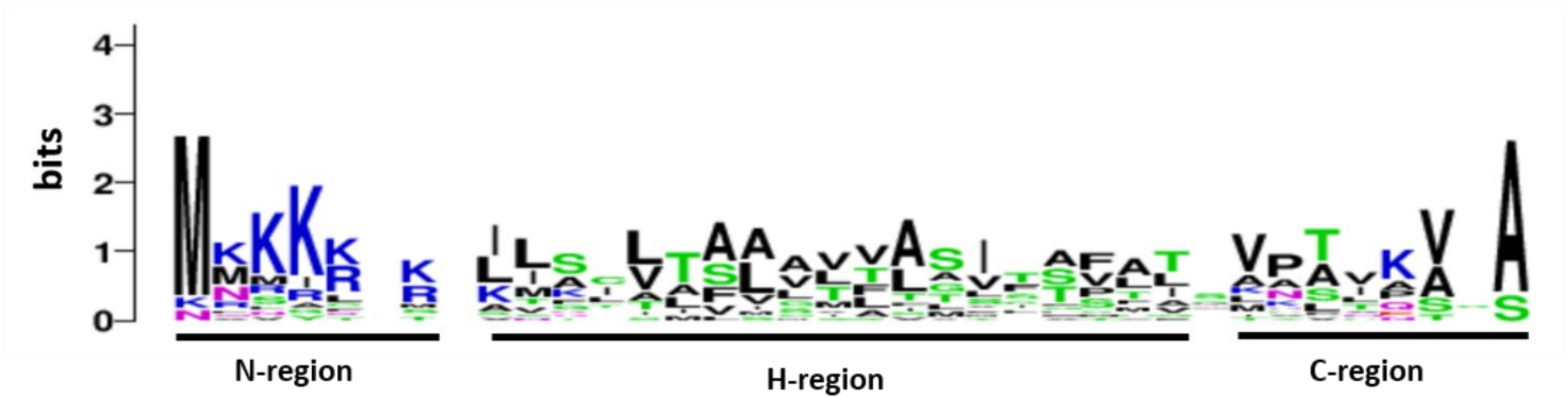

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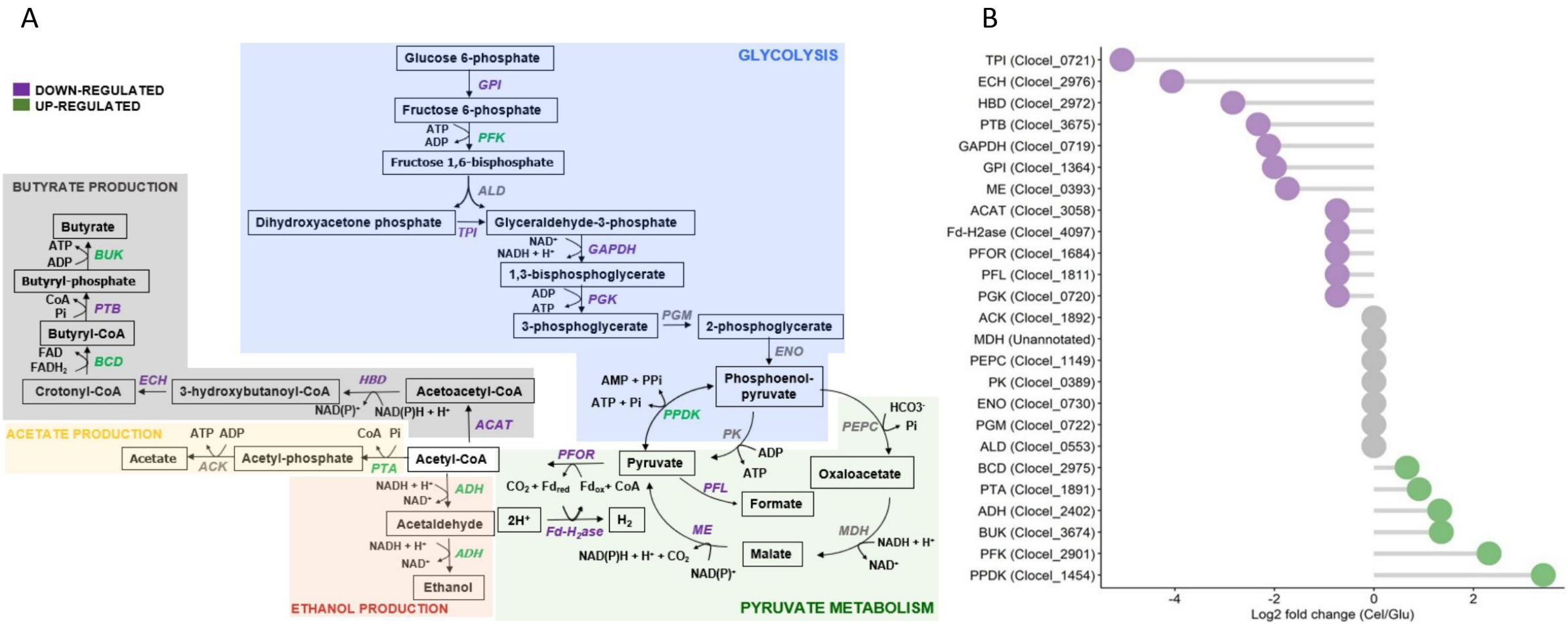

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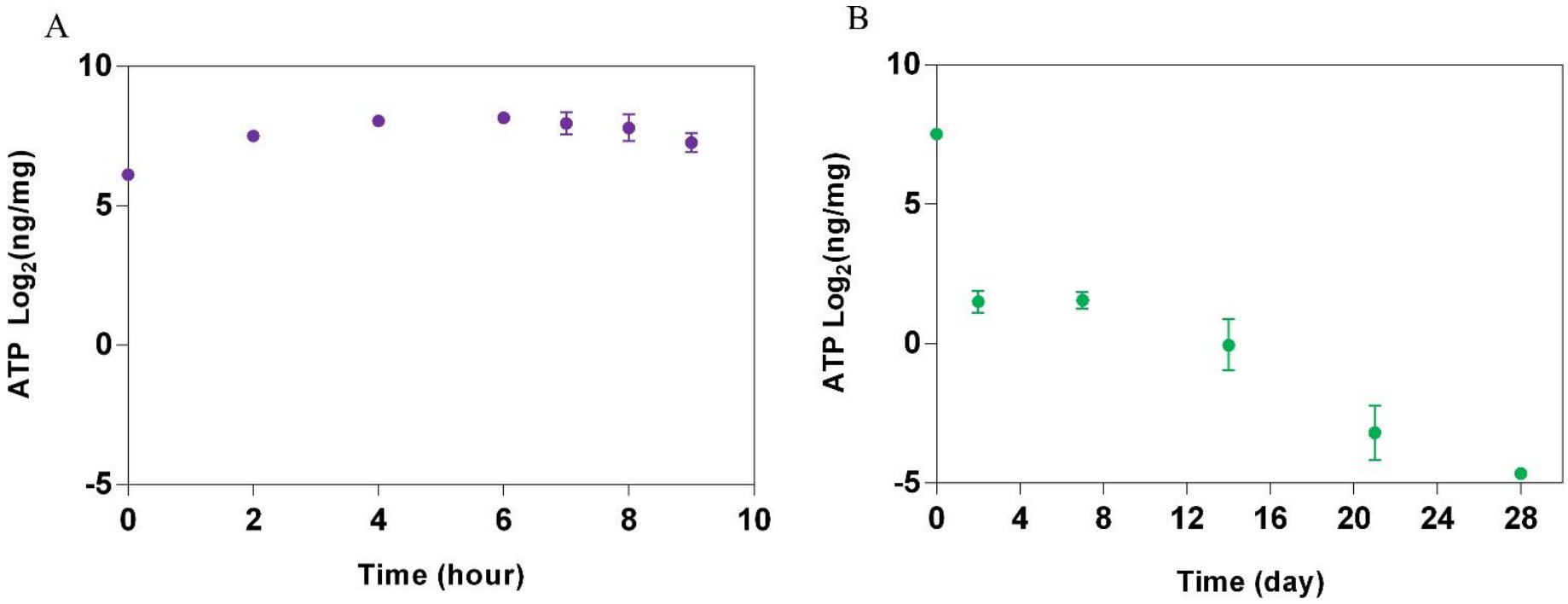

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